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Evaluation of the effects of selected phytochemicals on quorum sensing inhibition and *in vitro* cytotoxicity

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Abstract

Quorum sensing (QS) is an important regulatory mechanism in biofilm formation and differentiation. The interference with QS can affect biofilm development and antimicrobial susceptibility. This study evaluates the potential of selected phytochemical products to inhibit QS. Three isothiocyanates (allylisothiocyanate-AITC, benzylisothiocyanate-BITC and 2-phenylethylisothiocyanate-PEITC) and six phenolic products (gallic acid-GA, ferulic acid-FA, caffeic acid-CA, phloridzin-PHL, (-) epicatechin-EPI and oleuropein glucoside-OG) were tested. A disc diffusion assay based on the pigment inhibition of *Chromobacterium violaceum* CV12472 was performed. In addition, the mechanisms of QS inhibition (QSI) based on the modulation of *N*-acyl homoserine lactones (AHLs) activity and synthesis by the phytochemicals were investigated. The cytotoxicity of each product was tested on a cell line of mouse lung fibroblasts. AITC, BITC and PEITC demonstrated capacity for QSI by modulation of AHLs activity and synthesis, interfering with QS systems of *C. violaceum* CviI/CviR homologues of LuxI/LuxR systems. The cytotoxic assays demonstrate low effects on the metabolic viability of fibroblast cell line only for FA, PHL and EPI.

Keywords: biofilms; *Chromobacterium violaceum*; cytotoxicity; isothiocyanates; phenolics; phytochemicals; quorum sensing inhibition

Introduction

The emergence of multi-resistant bacteria to conventional antibiotics has increased worldwide (Levy 2001; McDermott et al. 2003; Andersson et al. 2010). This problem is apparently due to the natural selective pressure and to the indiscriminate use of antibiotics

48 (Monroe et al. 2000; Andersson 2003). Another disadvantage of the use of conventional
49 antimicrobials is their failure to treat infections caused by bacterial biofilms (Costerton et
50 al. 1999; Anderson et al. 2008). Bacteria in sessile state are more protected against host
51 defenses, and highly tolerant to antimicrobials compared to their planktonic counterparts
52 (Lewis 2001). Consequently, new strategies are required to target those pathogenic
53 microorganisms that are able to grow in biofilms (Landini et al. 2010; Saleem et al. 2010;
54 Jorge et al. 2012). The best strategy to control biofilms is by preventing their
55 development. Therefore, the cellular processes of biofilm formation, maintenance, and
56 dispersal are important targets for the discovery of new inhibitors (Landini et al. 2010).
57 One of these approaches involves the use of compounds that interrupt the bacterial
58 communication in biofilms, instead of simply killing the bacteria (Kaufmann et al. 2008;
59 Lee et al. 2011; Jakobsen et al. 2012).
60 Several Gram-negative pathogens use signaling molecules called autoinducers (AIs),
61 such as *N*-acyl homoserine lactones (AHLs), to mediate communication systems in a
62 process termed quorum sensing (QS) (Adonizio et al. 2006). The first signal molecule of
63 this type was identified from *Vibrio fischeri*, a marine symbiotic bacterium, which
64 induces luminescence in this species (Dickschat 2010). These signaling molecules are
65 synthesized by enzymes of the LuxI family (referred as AHL synthethases) and can bind
66 to transcription regulators of the LuxR family (Greenberg 2000). AIs are constantly being
67 produced and received at basal level by bacterial cells (Adonizio et al. 2006; Dobretsov
68 et al. 2013). When the cell density of a bacterial population increases, the AHLs
69 concentration also increases. The AIs produced by bacteria diffuse out and accumulate in
70 the surrounding environment, and once a threshold concentration has been reached
71 (quorum level), they diffuse back into the bacteria, and induce the expression of a set of
72 target genes, that consequently change the behavior of bacteria (Daniels et al. 2004).

73 Hence, using QS bacterial populations can change from acting as individual cells to
74 functioning in a concerted multi-cellular manner, controlling many physiological
75 functions, such as bioluminescence, pigment production, production of antimicrobial
76 compounds, conjugation, bacterial motility and exopolysaccharide synthesis (Whitehead
77 et al. 2001; Manefield et al. 2002; Kaufmann et al. 2008; Dobretsov et al. 2011).
78 Moreover, it has been shown that QS is an important regulatory mechanism in biofilm
79 formation/differentiation and in the expression of genes involved in processes related to
80 survival, virulence and pathogenicity (Hentzer et al. 2003a; Dickschat 2010; Landini et
81 al. 2010). Various pathogenic bacteria such as *Pseudomonas aeruginosa*, *Vibrio* spp.,
82 *Burkholderia cepacia* and *Yersinia enterocolitica* employed QS to regulate their virulence
83 and pathogenicity (Khan et al. 2009).

84 The understanding of the mechanisms underlying biofilm formation could lead to
85 approaches for their control and prevention. Biofilm formation is a dynamic and
86 multicellular process mediated by a combination of adhesion mechanisms,
87 exopolysaccharide (EPS) synthesis, bacterial motility and QS phenomenon that are
88 intrinsically related (Simões et al. 2009; Landini et al. 2010; Simões 2011). Several aspect
89 of biofilm dynamic, such as heterogeneity, architecture, stress resistance, maintenance
90 and sloughing have been documented to be mediated by signaling molecules. The role of
91 AHLs in the regulation of colonization events and in the differentiation of microcolonies
92 was also described by(Davies et al. 1998). Therefore, the interference with the
93 communication system of microorganisms is a promising target to tackle biofilms (Zhang
94 et al. 2004; Landini et al. 2010). The discovery that several products with anti-biofilm
95 activity (e.g., halogenated furanones from Australian macroalgae, *Delisea pulchra*) are
96 QS inhibitors demonstrated the importance of this signaling system in biofilm prevention
97 and control (Hentzer et al. 2002; Manefield et al. 2002; Persson et al. 2005; Rasmussen

98 et al. 2005b). Moreover, other studies verified that with the block of signaling molecules
99 it is possible to reestablish the action of the immune system and, therefore, eliminate the
100 infectious microorganism (Hentzer et al. 2003a; Hentzer et al. 2003b; Bjarnsholt et al.
101 2005). Biofilms constitute not only a medical problem but may also have negative
102 implications for the industry (food spoilage, pressure drop, biocorrosion, etc) and the
103 environment (aquaculture diseases, drinking water distribution systems, etc). This
104 microbial consortium is considered to be an initial step in the development of biofouling,
105 which develops in practically all natural and engineered aqueous systems. Biofouling is
106 a serious global problem in marine systems, causing extensive material and economic
107 costs worldwide. The interference with bacterial QS has been proposed as one approach
108 for controlling biofouling, due to the fact that compounds with capacity for QSI, affect
109 biofilm formation and have also implication in biofouling (Qian et al. 2009; Qian et al.
110 2013). Natural products from marine organisms, fungi, and aquatic/terrestrial plants have
111 shown to be effective inhibitors of biofouling due to their well-recognized capacity to
112 produce QS inhibitors and, consequently, to inhibit biofilm formation (Dobretsov et al.
113 2009; Qian et al. 2009; Dobretsov et al. 2011; Dobretsov et al. 2013).

114 The majority of QSI compounds characterized so far have not been qualified as
115 chemotherapeutic agents due to their toxicity, high reactivity and instability (Khan et al.
116 2009). Hence, new products with less harmful effects have a greater advantage for
117 humans, and the attention has been focused on identification of such compounds from
118 natural and sustainable sources. In recent years, some reports have been published about
119 natural compounds with capacity to inhibit QS, such as weeds, dietary products and
120 medicinal plant extracts (Gao et al. 2003; Vattem et al. 2007; Adonizio et al. 2008; Zhu
121 et al. 2008; Khan et al. 2009). Plants synthesize secondary metabolites (phytochemicals)
122 that are a fundamental source of chemical diversity and important components of the

123 current pharmaceutical products (Dixon 2001; Kubo et al. 2006; Saavedra et al. 2010;
124 Saleem et al. 2010). In this context, it is known that some dietary phytochemicals, such
125 as phenolics, glucosinolates (GLS) and their hydrolysis products, have beneficial health
126 properties (Prior et al. 2000; Aires et al. 2009a; Aires et al. 2009b). These properties
127 include, antibacterial, antiviral, antioxidant, anti-inflammatory, antiallergic and
128 anticarcinogenic activities, hepatoprotective and antithrombotic effects and vasodilatory
129 action. (Fahey et al. 2001; Soobrattee et al. 2005; Srinivasan et al. 2007; D'Antuono et al.
130 2009; Wang et al. 2010). Phenolic products, including simple phenolics, their derivatives
131 and complex flavonoids and tannins, are among the most important and abundant plant
132 secondary metabolites (Morton et al. 2000; Manach et al. 2004). These compounds can
133 be found in vegetable, fruits, chocolate and beverages (Soobrattee et al. 2005). Another
134 important group of phytochemicals, known for their health benefits, are the glucosinolates
135 hydrolysis products (GHP), particularly isothiocyanates (ITCs). These compounds can be
136 found in the *Brassicaceae* family (i.e. cabbage, broccoli, mustard, horseradish and
137 wasabi) and have long been recognized for their antimicrobial activity against clinical
138 important microorganisms (Fahey et al. 2001; Kim et al. 2009; Saavedra et al. 2010).
139 This study aims the assessment of the potential of nine selected phytochemicals
140 (phenolic products and ITCs) in QS inhibition (QSI) and the evaluation of their
141 cytotoxicity against a mouse lung fibroblast cell line in order to ascertain their potential
142 application in antimicrobial chemotherapy. The selection of phytochemicals was based
143 on previous studies performed with these compounds, which demonstrated good
144 antimicrobial activity against Gram-negative and positive bacteria in both planktonic and
145 sessile states (Saavedra et al. 2010; Borges et al. 2012; Borges et al. 2013a; Borges et al.
146 2013b).
147

148 **Material and methods**

149 ***Bacterial strains and culture conditions***

150 *Chromobacterium violaceum* CV12472 produces and responds to the AIs C6-AHL and
151 C4-AHL and was used to determine QSI activity. *C. violaceum* CV31532 (an
152 overproducer of AIs C6-AHL) and CV026 (a mini Tn-5 mutant of the wild strain
153 CV31532, which is not capable to produce its own AHL molecules, but responds to
154 exogenous active signal molecules C4-AHL and C6-AHL) were used to evaluate the
155 modulation of AHLs activity and synthesis (McClean et al. 1997; Choo et al. 2006). The
156 bacteria were routinely cultured aerobically in Luria–Bertani broth (LB; Liofilchen, Italy)
157 at 30 ºC with 150 rpm agitation in a shaking incubator (AGITORB 200, Aralab, Portugal),
158 prior to experiments. LB agar was used to test the activity of phytochemicals on QSI and
159 on modulation of AHL activity/synthesis.

160

161 ***Phytochemicals***

162 The phytochemicals selected for screening of QSI (Table 1), were: gallic acid (GA),
163 ferulic acid (FA), caffeic acid (CA), phloridzin (PHL), (-) epicatechin (EPI), oleuropein
164 glucoside (OG), allylisothiocyanate (AITC), benzylisothiocyanate (BITC) and 2-
165 phenylethylisothiocyanate (PEITC). All phytochemicals were obtained from Sigma-
166 Aldrich (Portugal), and their solutions were prepared with dimethyl sulfoxide (DMSO;
167 Sigma, Portugal). Controls were performed with DMSO and LB broth.

168

169

170 ***Bioassay for detection of quorum sensing inhibition***

171 Standard disc diffusion assay (Bauer et al. 1966) was performed with biosensor strain *C.*
172 *violaceum* CV12472 to detect QSI activity of the selected phytochemicals.

173 Concentrations lower, equal and higher than the MIC (minimum inhibitory concentration)
174 (5, 10, 15, 20, 25, 50, 75, 100, 112.5, 150, 200, 250, 300, 350, 375, 500, 750 and 1000 μg
175 ml^{-1}), that were previously determined by the microdilution method (Borges et al. 2012)
176 were tested. Briefly, LB agar (LB; Liofilchen, Italy) plates were inoculated with 100 μl
177 (1.4×10^8 CFU ml^{-1}) from an overnight culture of *C. violaceum* CV12472. Afterwards,
178 sterile paper disks (6 mm in diameter) (Oxoid, Spain) were placed over the plates and
179 were loaded with 15 μl of different concentrations of each phytochemical. DMSO and
180 LB broth were used as controls. In the biosensor strain *C. violaceum* CV12472 the
181 production of a purple pigment (violacein) is under control of a QS system that in turn is
182 mediated by the activity of AHLs (McClean et al. 1997; Chernin et al. 1998; Stauff et al.
183 2011). These signaling molecules are produced by the autoinducer synthase CviI, and
184 when a quorum has been reached, the AHLs bind to the transcriptional regulator CviR
185 and this complex triggers the expression of violacein production (Choo et al. 2006). After
186 24 h of incubation at 30 ºC, the inhibition of the pigment production around the disc (a
187 ring of colorless but viable cells) was checked. Antimicrobial activity is indicated by the
188 lack of microbial growth. Bacterial growth inhibition was measured as diameter 1 (d_1) in
189 mm while both bacterial growth and pigment inhibition were measured as a total diameter
190 2 (d_2) in mm. Thus, QSI, assessed by pigment inhibition, was determined by subtracting
191 the diameter of bacterial growth inhibition (d_1) from the total diameter (d_2) (QSI = d_2-d_1),
192 according to Zahin et al. (2010).

193 After the initial screening, using the qualitative agar discs diffusion method, QSI
194 caused by phytochemicals was also quantified in a broth assay, according to Choo et al.
195 (2006). With this purpose, *C. violaceum* CV12472 (OD_{620nm} = 0.1) supplemented with
196 different concentrations of phytochemicals were incubated at 30 ºC in a shaking incubator
197 (150 rpm) for 24 h. Then, violacein extraction was carried out. Briefly, 1 ml of culture

198 from each flask was centrifuged at 14549 *g* for 10 min to precipitate the insoluble
199 violacein and bacterial cells. The culture supernatant was discarded and 1 ml of DMSO
200 was added to the pellet. The solution was vortexed vigorously for 30 s to completely
201 solubilize violacein, and centrifuged at 14549 *g* for 10 min to remove the cells. Two
202 hundred microlitres of the violacein containing supernatants were added to 96-well
203 polystyrene microtiter plates and the absorbance was read with a microplate reader
204 (Spectramax M2e, Molecular Devices, Inc.) at a wavelength of 585 nm. The results were
205 expressed as percentage of violacein reduction. All tests were performed in triplicate with
206 three independent experiments.

207

208 ***Modulation of AHLs activity and synthesis***

209 The agar diffusion double ring assay was used to evaluate the effect of the phytochemicals
210 on the modulation of both AHLs activity and synthesis, as described previously (McLean
211 et al. 1997). For this test, only the phytochemicals that showed capacity for QSI in the
212 disc diffusion assay were used. After overnight growth, the cultures of *C. violaceum*
213 CV31532 and *C. violaceum* CV026, were adjusted to OD_{620nm} = 0.1 and subsequently
214 assayed for violacein production. Sterile paper disks were placed on the center of the LB
215 agar plates and were loaded with 15 µl of different phytochemical concentrations.
216 Controls with DMSO and LB broth were performed. To test for AHLs activity, the
217 biosensor *C. violaceum* CV026 was streaked in a circle on the LB agar plates in close
218 proximity to the tested phytochemical and the overproducer *C. violaceum* CV31532 was
219 streaked on the outside (4-5 mm of distance between two biosensors). To assess the
220 inhibition of AHLs synthesis, the location of AHLs overproducer and biosensor strains
221 was inverted. Three independent assays were performed.

222

223 ***Cell line and culture conditions***

224 The cell line of mouse lung fibroblasts (L929), obtained from the European Collection of
225 Cell Cultures (ECACC) was selected for cytotoxicity studies. The cells were grown in
226 Dulbecco's Modified Eagle Medium (DMEM; Sigma, Portugal), supplemented with 10%
227 of fetal bovine serum (FBS; Biochrom, Germany), 1% of antibiotics penicillin-
228 streptomycin (PEN-STREP; Invitrogen) and 3.7 g l^{-1} of sodium bicarbonate, and
229 incubated at 37 ºC in a humidified atmosphere containing 5% of CO_2/95% air, prior to
230 the experiments.

231

232 ***Cytotoxicity screening***

233 In order to evaluate the *in vitro* cytotoxicity of the selected phytochemicals, the cells were
234 grown in DMEM with FBS and antibiotics (penicillin and streptomycin, Sigma), and
235 trypsinized before the experiments, according to ISO/EN 10993 (part 5) guidelines with
236 some modifications (ISO document 10993 1992). Then, the cell line was seeded at an
237 appropriate density (1×10^4 cells/well) in 96-well polystyrene microtiter plates (5 wells
238 per tested condition) and allowed to attach for 24 hours at 37°C, in a humidified
239 atmosphere containing 5% of CO_2. After 24 h of cell seeding, culture medium was
240 discarded and replaced by 200 µl of fresh DMEM with each phytochemical at several
241 concentrations (50, 100, 500 and 1000 µg ml^{-1}) that were prepared with DMSO, and
242 incubated for 72 h at 37 ºC in a humidified atmosphere containing 5% of CO_2. The
243 phytochemicals did not exceed 1.5% (v/v) of the well final volume. Cells with DMSO
244 (1.5%, v/v) and without phytochemicals were used as controls. The cell viability was
245 evaluated by MTS assay (Baran et al. 2004). Three independent experiments were
246 performed.

247

248 ***Cell viability assay***

249 After 72 h of exposure to phytochemicals, cell viability was assessed using Cell Titer 96®
250 One solution Cell Proliferation Assay kit (Promega, USA). In this test, the substrate MTS
251 (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-
252 tetrazolium) is bioreduced into a brown formazan product by dehydrogenase enzymes in
253 metabolically active cells (Baran et al. 2004). The phytochemicals were removed with a
254 micropipette and 200 µl of MTS solution, prepared with DMEM with antibiotics and
255 without FBS in a 5:1 ratio, was added into each well. Cells were then incubated for 3 h at
256 37º C in a humidified atmosphere containing 5% of CO_2 in the dark. The optical density
257 of each well was measured at 490 nm using a microplate reader. The results were
258 expressed as percentage of cell viability.

259

260 ***Statistical analysis***

261 The data were analysed using the statistical program SPSS version 20.0 (Statistical
262 Package for the Social Sciences). The mean and standard deviation within samples were
263 calculated for all cases. At least three independent experiments were performed for each
264 condition tested. One-way Anova with Bonferroni test was used to assess the statistical
265 significance value (confidence level ≥ 95%). $P < 0.05$ was considered statistically
266 significant.

267

268 **Results**

269 The MICs of the tested phenolics and ITCs against biosensor strain *C. violaceum*
270 CV12472 are presented in Table 1. The MIC of all phenolics tested was > 1000 µg ml^{-1}.
271 BITC had the lowest MIC (10 µg ml^{-1}) while AITC and PEITC had a MIC of 15 µg ml^{-1}.

272 A disc diffusion assay was performed for QSI screening using the biosensor strain *C.*
273 *violaceum* CV12472. A range of concentrations (from sub-MICs to > MICs) were tested
274 in order to ascertain if the halos produced around the disks were due to growth inhibition
275 of cells and/or QSI (see Supplementary material). Hence, loss of purple pigment by *C.*
276 *violaceum* CV12472 is indicative of QSI by the phytochemicals. Amongst the nine
277 phytochemicals screened, no pigment inhibition was observed with phenolics at the tested
278 concentrations. However, growth inhibition halos between 8 and 12 mm were detected
279 (data not shown). GA and FA at concentrations between 5 and 100 μg ml^{-1} produced halos
280 of growth inhibition of 8 mm and, for higher concentrations, the halos size was around
281 11 and 12 mm. Other phenolics, CA, OG, PHL and EPI, produced halos of growth
282 inhibition of 10 mm for all the concentrations tested (data not shown). QSI was only
283 detected for the three ITCs (Table 2). AITC at concentrations between 5 and 200 μg ml^{-1}
284 caused halos of growth inhibition of 10 and 11 mm, and no activity on pigment inhibition
285 was observed. Concentrations of 250, 300 and 350 μg ml^{-1} caused partial pigment
286 reduction in addition to antimicrobial activity (halos of 14 mm). AITC caused QSI halos
287 of 5 (350 μg ml^{-1}), 14 (375 and 500 μg ml^{-1}) and 45 mm (750 and 1000 μg ml^{-1}). For these
288 cases, the white zones of inhibition were opaque and not transparent, indicating that the
289 halo observed was caused by inhibition of violacein production, and not due to cell growth
290 inhibition. Moreover antimicrobial activity was also observed in addition to QSI,
291 producing halos of growth inhibition around the disks of 14 (350 μg ml^{-1}), 20 (375 μg ml-
292 1), 24 (500 μg ml^{-1}), and 40 mm (750 and 1000 μg ml^{-1}) of diameter (Table 2). For BITC
293 at 5 and 10 μg ml^{-1}, no pigment inhibition was observed, but it was detected antimicrobial
294 activity with halos of 10 and 12 mm, respectively (Table 2). For concentrations between
295 15 and 250 μg ml^{-1}, it was observed antimicrobial activity (inhibition zones of 20-74 mm)
296 and also QSI (halos ranging from 5 to 13 mm). For concentrations higher than 250 μg ml-

297 ^{1}, the growth of the biosensor strain was completely inhibited. For PEITC, clear halos
298 indicating the absence of growth of the biosensor microorganism were observed for
299 concentrations between 5 and 200 μg ml^{-1}. For all concentrations higher than 200 μg ml^{-}
300 ^{1}, both QSI (halos ranging from 5 to 35 mm) and growth inhibition (inhibition halos of
301 35-50 mm) were observed.

302 In order to evaluate the extent of QSI, the extraction and quantification of violacein
303 from CV12472 cultures in the absence and presence of phytochemicals at different
304 concentrations were also performed. Figure 1 shows the results of violacein inhibition by
305 ITCs (%). Violacein production was inhibited more than 85% with AITC for all
306 concentrations tested, except for the concentration of 5 μg ml^{-1} (70%). For concentrations
307 between 10 and 1000 μg ml^{-1}, no increase of violacein inhibition was observed ($P < 0.05$).
308 The same behavior was verified with BITC. For concentrations higher than 15 μg ml^{-1},
309 the percentage of violacein inhibition was around 90%. At 5, 10 and 15 μg ml^{-1} the
310 percentage of violacein inhibition with BITC was 81, 84 and 86%, respectively. For
311 PEITC, violacein production was inhibited more than 80% for concentrations higher than
312 75 μg ml^{-1}. A maximum of 89% inhibition in violacein production was observed with
313 PEITC at 1000 μg ml^{-1} (Figure 1). For the smaller concentrations, the percentage of
314 violacein inhibition was 47, 57, 60, 76, 77 and 79%, for concentrations of 5, 10, 15, 20,
315 25 and 50 μg ml^{-1}, respectively. Since QSI was not observed with the phenolic compounds
316 by the disk diffusion method, only the highest concentration used was selected for broth
317 assays. The percentage of violacein inhibition by phenolics at 1000 μg ml^{-1} was: 75 (CA),
318 72 (FA), 59 (GA), 51 (OG), 48 (PHL) and 33% (EPI) (Figure 2).

319 The effects of ITCs (which had positive results for QSI) on modulation of AHLs
320 activity and synthesis was also performed. For this assay, a range of concentrations above
321 and below the one that inhibited the violet pigment were selected. The AHLs activity was

322 assessed by the decrease of the violacein pigment production in *C. violaceum* CV026
323 AHL biosensor, due to low levels of QS related with AHL detection. The AHLs synthesis
324 was also evaluated by pigment inhibition of CV026, due to the decrease or absence of
325 AHLs production by overproducer *C. violaceum* CV31532 in the presence of the
326 phytochemicals. Our results demonstrated that AITC had capacity to interfere with the
327 activity of the AHLs produced by CV31532. This effect started at 25 µg ml^{-1}, causing a
328 small decrease in the violacein production which was intensified at 100 and 250 µg ml^{-1}
329 (data not shown). No pigment was observed for concentrations higher than 350 µg ml^{-1}
330 (data not shown). For BITC, pigment inhibition of CV026 was verified for concentrations
331 between 2 and 5 µg ml^{-1} (data not shown). Total inhibition was observed for the higher
332 concentrations. PEITC also showed positive interference with AHLs activity at 20 µg ml^{-}
333 ^{1} and no pigment of CV026 was found for concentrations above 50 µg ml^{-1} (data not
334 shown). The results on the ability of ITCs to reduce the production of AHLs molecules
335 from CV31532 were identical to AHLs activity (data not shown).

336 The cytotoxicity of the selected phytochemicals was tested on a cell line of mouse lung
337 fibroblasts (L929) (Figure 3). L929 cells produced large amounts of a brown formazan
338 product after 72 h of exposure to PHL for all concentrations tested. The viability after
339 exposure to this phenolic was statistically equal to the control ($P > 0.05$) except for the
340 concentration of 1000 µg ml^{-1} ($P < 0.05$). FA at 50 and 100 µg ml^{-1} also produced higher
341 quantity of formazan with a percentage of viable cells of 98 and 95%, respectively ($P >$
342 0.05). Viabilities of 96 and 95% were obtained after exposure to EPI at 50 and 100 µg
343 ml^{-1}, respectively. With these compounds at 500 µg ml^{-1} (FA – 67%; EPI – 17%) and
344 1000 µg ml^{-1} (FA – 28%; EPI – 30%), it was verified a decrease in the percentage of
345 viable cells ($P < 0.05$). The percentage of viable cells with OG at 50 µg ml^{-1} was 57%.
346 The increase of OG concentration reduced significantly the cells viability ($P < 0.05$). The

347 ITCs reduced significantly the viability of the L929 cells ($P < 0.05$), being the toxicity
348 levels similar for the diverse concentrations tested ($P > 0.05$). The same behavior was
349 verified with GA and CA ($P < 0.05$).
350

351 **Discussion**

352 The occurrence of planktonic and sessile bacteria, resistant to antimicrobials, increases
353 the need for the development of new strategies to control microbial growth (Adonizio et
354 al. 2006; Khan et al. 2009). More knowledge about QS systems allowed the employment
355 of new approaches for the development of drugs to control bacterial infections by
356 different mechanisms from that of actual antibiotics. So, the disruption of the QS
357 signaling pathways can help to overcome the bacterial resistance problem and are a
358 possible key to treat infections caused by biofilms (Dickschat 2010; Zhu et al. 2011;
359 Jakobsen et al. 2012; Qian et al. 2013).
360 In the present study, phenolic compounds and ITCs, that are commonly present in
361 dietary products, were evaluated as potential QS inhibitors. These compounds are thought
362 to be an integral part of both human and animal diets. Preliminary reports showed that
363 some of these phytochemicals have antimicrobial properties with potential for biofilm
364 prevention and control (Borges et al. 2012; Borges et al. 2013b).
365 The results of the qualitative screening of nine phytochemicals indicated that all ITCs
366 tested (AITC, BITC and PEITC) have potential QSI activity based on *C. violaceum*
367 pigment production. In fact, ITCs had antimicrobial activity in addition to QSI. For these
368 compounds, two halos were observed: the first was a clear halo (bacterial growth
369 inhibition), demonstrating antimicrobial activity; the second was opaque (bacterial
370 growth without violet pigment production) and corresponds to QSI. Similar results were
371 observed in other studies, with extracts and essential oils from medicinal plants (Adonizio

372 et al. 2006; Khan et al. 2009; Koh et al. 2011). This QSI may be associated to the
373 antimicrobial activity of phytochemicals, as proposed by Skindersoe et al. (2008)

374 The analysis of the ITCs structure and their QSI shows a higher potential of the
375 aromatic GHP (BITC and PEITC) relatively to aliphatic GHP (AITC). In addition, BITC
376 was the most effective ITC for QSI, acting at low doses (15 up to 250 μg ml^{-1}). However,
377 the QSI activity verified with the ITCs tested needs to be further characterized regarding
378 the genes that are affected by the phytochemicals.

379 The QS systems of *C. violaceum* consist of CviI/CviR that are homologues of
380 LuxI/LuxR systems. This strain mediates QS by AIs of the type of AHLs (C4-AHL and
381 C6-AHL) (Blosser et al. 2000; Morohoshi et al. 2008; Stauff et al. 2011). These molecules
382 are AHL-based QS systems synthesized by LuxI-type AHL synthases and detected by
383 their cognate LuxR-type receptors. Based on the fact that the ITCs tested in this study
384 inhibit QS systems of *C. violaceum*, and for the high similarity of QS systems that use the
385 same type of AHLs signaling molecules (Dickschat 2010), it is possible that these
386 compounds can interfere with other homologues QS systems. Additional studies with
387 other biosensors that use different QS systems are need to confirm this possibility.

388 Phenolic compounds inhibited the growth of the bacterium but not the pigment
389 production. The lack of QSI detection of the tested phenolics can be due to the limitations
390 of QSI assay used. These compounds can affect QS systems in a different way from that
391 of *C. violaceum.* Indeed, Huber et al. (2003) found that some polyphenolic compounds
392 having a GA moiety [(-)-epigallocatechin gallate, (+)-catechin and tannic acid] shown
393 positive interference with bacterial QS of *Escherichia coli* and *Pseudomonas putida*.
394 Liang et al. (2009) observed that salicylic acid showed activity anti-QS against *P.*
395 *aeruginosa* in a dose-dependent manner. Extracts from *Moringa oleifera*, which contains

396 phenolics compounds such as GA, chlorogenic acid and quercetin in appreciable
397 quantities demonstrated QSI potential (Singh et al. 2009).

398 The common mechanisms of QS interference include: (i) inhibition of signal
399 biosynthesis or inhibition of activity of AHLs-producing enzymes, (ii) enzymatic signal
400 degradation, and (iii) inhibition of reception signal molecules (Zhang et al. 2004;
401 Rasmussen et al. 2006b, a; Khan et al. 2009). In this study, it was investigated the effects
402 of ITCs on modulation of AHLs activity (*via* LuxR-type receptor) and synthesis (*via*
403 LuxI-type AHL synthases). All ITCs revealed capacity to modulate both AHLs activity
404 and synthesis. The results of CV31532/CV026 systems also indicate that BITC was the
405 most potent ITC based on pigment inhibition of CV026 strain. Based on both results of
406 disc diffusion and double ring assays it is possible to infer that QSI in the *C. violaceum*
407 systems, with ITCs, can be mediated by the interference with synthesis or activity of AIs
408 C6-AHLs. Therefore, QSI can be due to a combination of two mechanisms: interference
409 with the activity of AHLs produced by CV31532 (LuxR system homologue) and
410 modulation of the synthesis of AHLs by CV31532 (LuxI system homologue) as detected
411 by the extension of violacein production by the AHLs biosensor CV026. This result was
412 also found by Vattem et al. (2007) with dietary phytochemicals. Chenia (2013) achieved
413 the same result with *Kigelia africana* extracts.

414 QSI by ITCs can be achieved by several ways: the ITCs could affect the QS signaling
415 cascade of CV31532 strain, binding directly to the LuxR-type receptor by competing with
416 the AHLs molecules and/or by preventing the binding of the AHLs molecules to these
417 receptors (Hentzer et al. 2003a; Rasmussen et al. 2005a; Rasmussen et al. 2005b). These
418 phytochemicals could also affect the ability of CV31532 to synthesize AHLs by
419 decreasing the expression of the LuxI-type synthase, which synthesizes the AHLs
420 molecules. Decreased AHLs synthesis may also be explained by the ability of these

421 phytochemicals to inhibit the LuxI-type enzyme activity (Schauder et al. 2001). Since the
422 ITCs were effective at inhibiting QS mediated by two different AHLs producers
423 (CV12472 and CV31532), it may be assumed that these compounds are able to inhibit
424 multiple bacterial QS systems homologues of LuxI/LuxR systems which are mediated by
425 AHLs molecules.

426 Although the disc diffusion method is effective for the detention of inhibitors of QS,
427 it is not possible to know the exact quantities of violacein inhibition. This drawback
428 indicates the need for an assay that can measure the amount of violacein production. With
429 the aim of reinforcing the results obtained in the qualitative assay related to pigment
430 inhibition, violacein was extracted and quantified, after 24 h of exposure to
431 phytochemicals. The results showed a percentage of violacein inhibition higher than 70%
432 for ITCs regardless the concentration used, except for PEITC at the lowest concentrations
433 (5, 10 and 15 μg ml^{-1}). The results obtained by broth studies also demonstrated significant
434 inhibition of the percentage of violacein production by phenolic compounds. Although,
435 in disc diffusion assay no positive result for QSI was observed. This is apparently due to
436 inhibition of microbial growth and not violacein synthesis. Another possibility for the
437 observed results with the phenolics is the alteration of the permeability of the cell
438 membrane that influence the flux of AHLs, as verified by Skindersoe et al. (2008). In fact,
439 phenolic compounds, such as GA and FA, can interfere with the membrane permeability
440 of some Gram-negative and positive bacteria, as demonstrated in a previous study
441 (Borges et al. 2013a).

442 The use of QS inhibitors could have the potential to circumvent the problem of
443 bacterial resistance (Cady et al. 2012). Since reports on dietary phytochemicals as source
444 of QS modulators are scarce, the results obtained in this work with ITCs are relevant. The
445 presence of such compounds in natural foods is extremely interesting because, in most

446 cases, vegetables are nontoxic to humans and readily available. Additionally, functional
447 foods with beneficial effects for health have attracted the attention of researchers and can
448 be used as a prophylactic treatment. Natural products with capacity for QSI can be used
449 with antibiotics as adjuvants in order to increase the susceptibility of resistant bacteria
450 (Cady et al. 2012). It was demonstrated by Brackman et al. (2011) that QSI increased the
451 susceptibility of bacterial biofilms to multiple types of antibiotics.

452 In order to know whether phenolic compounds or ITCs constitute pharmaceutically
453 relevant drug candidates, it was investigated the cytotoxic effects on mammalian cells,
454 using a cell line L929 that is routinely used in, *in vitro*, cytotoxicity assessments. The
455 results showed that 72 h after contact with PHL (at all concentrations), FA (at 50 and 100
456 $\mu g\ ml^{-1}$) and EPI (at 50 and 100 $\mu g\ ml^{-1}$), the majority of cells are viable. This supports
457 the absence of cytotoxic effects of these compounds and suggests their possible usage for
458 therapeutic applications. The addition of 50 $\mu g\ ml^{-1}$ of OG was sufficient to reduce about
459 50% of the cellular viability. Concentrations higher than 50 $\mu g\ ml^{-1}$ significantly
460 decreased the percentage of viable cells. The same result was observed with GA, CA and
461 with the three ITCs used, for all the concentrations tested. It is important to refer that
462 these compounds had a concentration-dependent effect on the viability of cells, except
463 CA. The increase of CA doses had a positive effect on the viability of the cells, *i.e.* higher
464 CA dose were less toxic to the cells. In fact, it is recognized that CA stimulates cell
465 proliferation at high doses (Ito et al. 1993; Kagawa et al. 1993; Lutz et al. 1997). Although
466 GA, FA and CA are phenolic acids, these compounds demonstrated different cytotoxicity.
467 This is apparently due to differences in their structure. The properties of phenolic products
468 vary according to the type of substituents, and with the number and positions of the
469 hydroxyl groups on the aromatic ring (Robbins 2003; Sroka et al. 2003; Stalikas 2007).
470 GA demonstrated to be more cytotoxic than CA and FA. This is probably due to the fact

471 that GA has more hydroxyl groups than CA and FA and, consequently, can interact more
472 strongly with the cells. In the same way, CA (with 2 hydroxyl groups) is more cytotoxic
473 than FA (with only 1 hydroxyl group). Nevertheless, the relationship between the
474 structural and cytotoxic differences of GA, FA and CA needs to be further elucidated. In
475 a work performed by Galati et al. (2006), it was verified that the major cytotoxic
476 mechanism found by GA for hepatocytes was mitochondrial membrane potential collapse
477 and reactive oxygen species (ROS) formation. Likewise, these cytotoxic effect and ROS
478 formation was increased by the presence of some metals such as copper, and enzymes
479 (NAD(P)H:quinone oxidoreductase 1). But these authors also demonstrated that
480 formation of an iron complex and glutathione (GSH) conjugation play an important role
481 in the detoxification of GA. So, the exposure of cells to GA in the presence of iron can
482 reduce the cytotoxic effect of GA as verified in this study. However, more tests are needed
483 to determine the additional aspects of the cytotoxicity of this compound.

484 The percentage of viable cells after the contact with the ITCs was low. It is known that
485 the differences in the activity of ITCs are dependent of the intracellular uptake (Zhang
486 2000, 2001). GSH may be involved in the uptake of ITCs, occurring mainly in the form
487 of GSH-conjugated dithiocarbamates, catalyzed by glutathione transferases (GST)
488 (Zhang 2000, 2001). So, the accumulation of ITCs is related to intracellular GSH levels.
489 Nevertheless, the degree of such synergism could depend on the type of ITC. For AITC,
490 BITC and PEITC, this synergism may be sometimes limited (Zhang 2001; Fimognari et
491 al. 2004). Therefore, it is possible to hypothesize that the cytotoxicity observed in this
492 study for these ITCs can be in part, but not totally, due to this mechanism. Moreover,
493 their high cytotoxic effects may be attributed to the reactive of the ITC group, which can
494 react with proteins and other crucial biomolecules. This reactivity can lead to the
495 formation of oxygen and other free-radicals that might cause cellular injuries. However,

496 *in vitro* studies do not necessarily predict *in vivo* outcomes. It is known that ITCs are
497 unstable and reactive in physiological environments. According to *in vivo* studies
498 performed by Amara et al. (2009), the reactive ITC group may react with host-encoded
499 proteins and therefore eliminate its bioactivity. Nevertheless, epidemiological studies
500 indicate that consumption of cruciferous vegetables reduce the risk of diverse human
501 cancers due to anticarcinogenic properties. There are a significant number of reports
502 indicating that ITCs can exert a cytotoxic action against tumor cells perturbing several
503 steps in the carcinogenic process (inhibition of cell growth, induction of apoptosis, DNA
504 fragmentation and cell death) (Xu et al. 2000; Fimognari et al. 2004; Zhang 2010).

505 Although the cytotoxicity observed in this work with the ITCs tested, the European
506 Food Safety Authority (EFSA) Panel on Food Additives and Nutrient Sources added to
507 Food (ANS) concluded in a recent report about the safety of AITC for the use as a food
508 additive, that no significant safety concerns are predictable with its use as anti-spoilage
509 agent (EFSA 2010). Moreover, ITCs products are promising candidates for food industry
510 due to their recognized antimicrobial activity against foodborne pathogens, and because
511 they do not influence the organoleptic properties of processed food (Delaquis et al. 1995;
512 Aires et al. 2009a; Al-Gendy et al. 2010). In a recent report of Mushantaf et al. (2012),
513 AITC was suggested as a possible disinfectant for non-potable water treatment. The
514 relatively immediate aqueous degradation of AITC is an advantage because it will not
515 persist in the environment (Gómez De Saravia et al. 1998; Mushantaf et al. 2012). Based
516 on these reports, AITC can be also a potential candidate to prevent biofouling and
517 microbial diseases in aquaculture.

518 In conclusion, AITC, BITC and PEITC were found to have QSI activity. These ITCs
519 affected the QS system of *C. violaceum* (CviI/CviR system - LuxI/LuxR homologue) not
520 only interfering with AHLs activity but also by modulating AHLs synthesis. These

521 phytochemicals were highly cytotoxic for the cell line of mouse lung fibroblasts. The
522 phenolic compounds tested did not showed QSI potential, apparently due to the fact that
523 they can interfere with other QS systems, not covered by the assay used in this study. The
524 initial *in vitro* antimicrobial and cytotoxic tests suggest the potential use of FA, PHL and
525 EPI as therapeutic antimicrobials. These results are particularly important in the case of
526 FA, due to its antimicrobial activity against strains with pathogenic potential in both
527 planktonic and biofilm states (Borges et al. 2012; 2013a), and to potentiate the action of
528 antibiotics (Saavedra et al. 2010).

529

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810

811 Table 1. Subgroups of phenolic and glucosinolate compounds, their chemical structure, dietary source, biological properties and MIC values against *C. violaceum* CV12472.

Phytochemical Name	Chemical class	Chemical structure	Dietary source	Biological properties	References	MIC ($\mu g\ ml^{-1}$)
Gallic acid (GA) (3,4,5-trihydroxybenzoic acid)	Phenolic acid (hydroxybenzoic acid)		Grapes (grape seeds), berries, tea leaves, oak bark.	Antioxidant Antibacterial; Anti-inflammatory; Antiallergic; Hepatoprotective; Antithrombotic; Antiviral; Anticarcinogenic; Vasodilator.	(Cushnie et al. 2005; Soobrattee et al. 2005; Boudet 2007; Srinivasan et al. 2007; Cueva et al. 2010).	NS
Ferulic acid (FA) (4-hydroxy-3-methoxy cinnamic acid)	Phenolic acid (hydroxycinnamic acid)		Cereals (corn flour, grain wheat, rice and oat flours).			NS
Caffeic acid (CA) (3,4-dihydroxycinnamic acid)	Phenolic acid (hydroxycinnamic acid)		Many fruits and vegetables (coffee).			NS
Phloridzin (PHL) (3,5-Dihydroxy-2-[3-(4-hydroxyphenyl)propanoyl]phenyl β-D-glucopyranoside)	Flavonoids (Chalcone glucoside)		Apples			NS
(-) Epicatechin (EPI) (2-(3,4-Dihydroxyphenyl)-3,4-dihydro-2H-1-benzo- pyran-3,5,7-triol)	Flavonoids (flavanols)		Many fruits, cocoa, nuts.			NS
Oleuropein Glucoside (OG) (2-(3,4-Dihydroxyphenyl)ethyl [2S-(2alpha,3E,4beta)]-3-ethylidene-2-(beta-D-glucopyranosyloxy)-3,4-dihydro-5-(methoxycarbonyl)-2H-pyran-4-acetate)	Flavonoids (*seco*-iridoid)		Olives (bark and leaves).			NS
Allylisothiocyanate (AITC) (3-Isothiocyanato-1-propene)	GHP (isothiocyanate)		*Brassica* Species (cabbage, broccoli, cauliflower).	Antimicrobial; Antioxidant; Anticarcinogenic.	(Fahey et al. 2001; Shin et al. 2004; Hong et al. 2008; D'Antuono et al. 2009; Saavedra et al. 2010; Wang et al. 2010; Dufour et al. 2012).	15
Benzylisothiocyanate (BITC) (Isothiocyanatomethylbenzene)	GHP (isothiocyanate)		Garden cress and *Brassica* Species.			10
2-Phenylethylisothiocyanate (PEITC) (2-Isothiocyanatoethylbenzene)	GHP (isothiocyanate)		Watercress and *Brassica* species.			15

812 (NS) – Non-susceptible, the MIC is higher than the maximum concentration tested (> 1000 µg ml^{-1}).

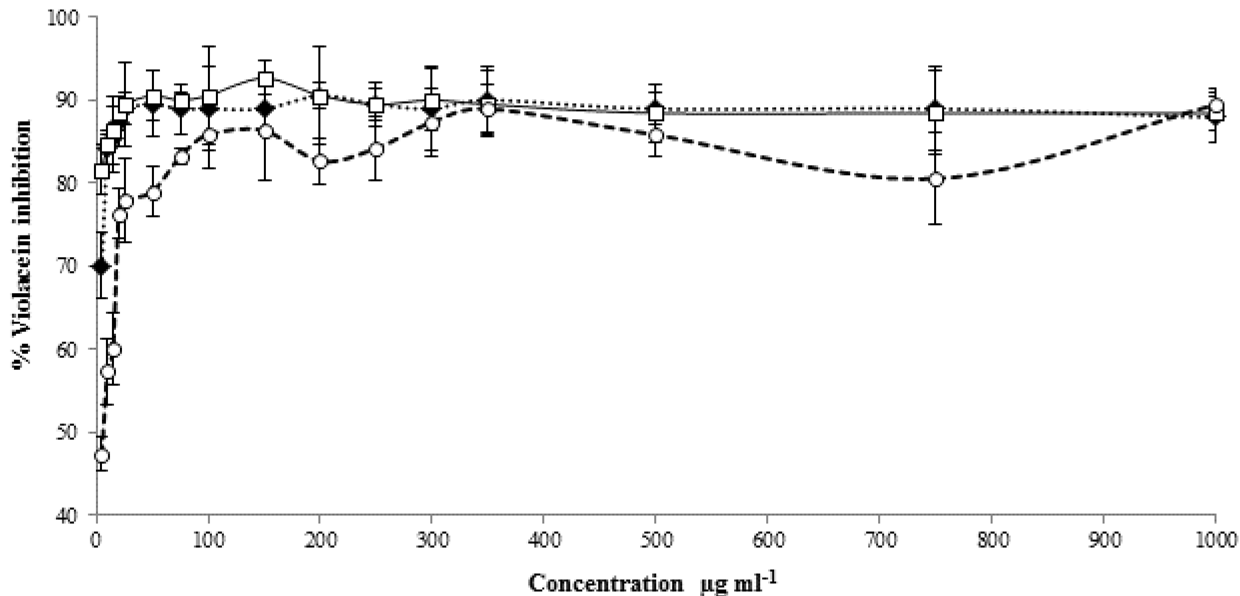
813 Table 2. Screening of ITCs that exhibited QSI and antimicrobial activity against *C. violaceum* strain CV12472

	AITC			BITC			PEITC		
[Phytochemical μg ml^{-1}]	Pigment production	AM[a] (mm)	QSI[b] (mm)	Pigment production	AM[a] (mm)	QSI[b] (mm)	Pigment production	AM[a] (mm)	QSI[b] (mm)
Medium	+	0	0	+	0	0	+	0	0
DMSO	+	0	0	+	0	0	+	0	0
5	+	10	0	+	10	0	+	11	0
10	+	10	0	+	12	0	+	11	0
15	+	11	0	+	20	8	+	15	0
20	+	10	0	+	20	8	+	15	0
25	+	11	0	+	20	8	+	15	0
50	+	10	0	+	20	8	+	22	0
75	+	10	0	+	54	6	+	27	0
100	+	10	0	+	52	13	+	30	0
112,5	+	11	0	+	65	12	+	33	0
150	+	11	0	+	65	10	+	33	0
200	+	11	0	+	74	5	+	33	0
250	+/-	14	0	+	74	5	-	35	5
300	+/-	14	0	t.i	t.i	t.i	-	40	10
350	+/-	14	5	t.i	t.i	t.i	-	40	10
375	-	20	14	t.i	t.i	t.i	-	40	30
500	-	24	14	t.i	t.i	t.i	-	40	30
750	-	40	45	t.i	t.i	t.i	-	50	35
1000	-	40	45	t.i	t.i	t.i	-	50	35

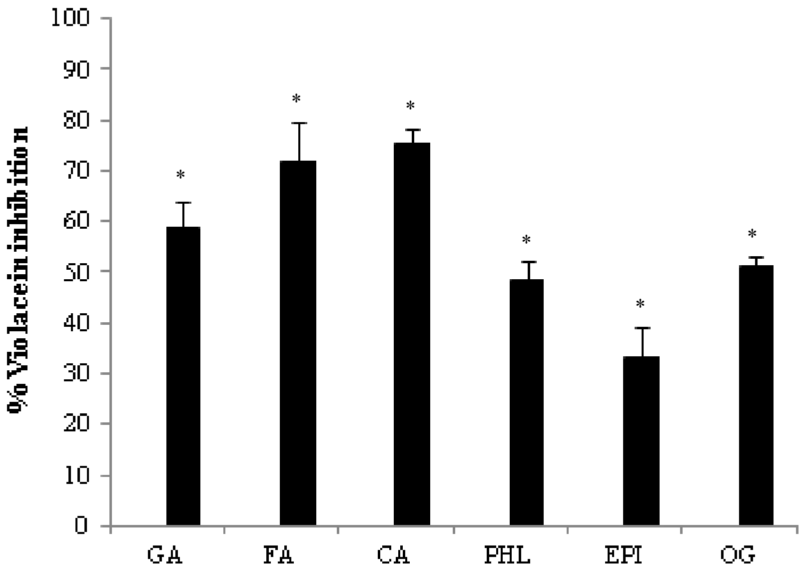
814 (+) – Visualization of pigment color; (-) – Absence of pigment color; (+/-) – Partial visualization of pigment color; (t.i) – Total inhibition (without grow).

815 [a] Antimicrobial activity observed as a clear halo, presented as diameter of zone of inhibition in millimeter.

816 [b] QSI observed as a halo of colorless with viable cells, presented as diameter of QSI in millimeter.

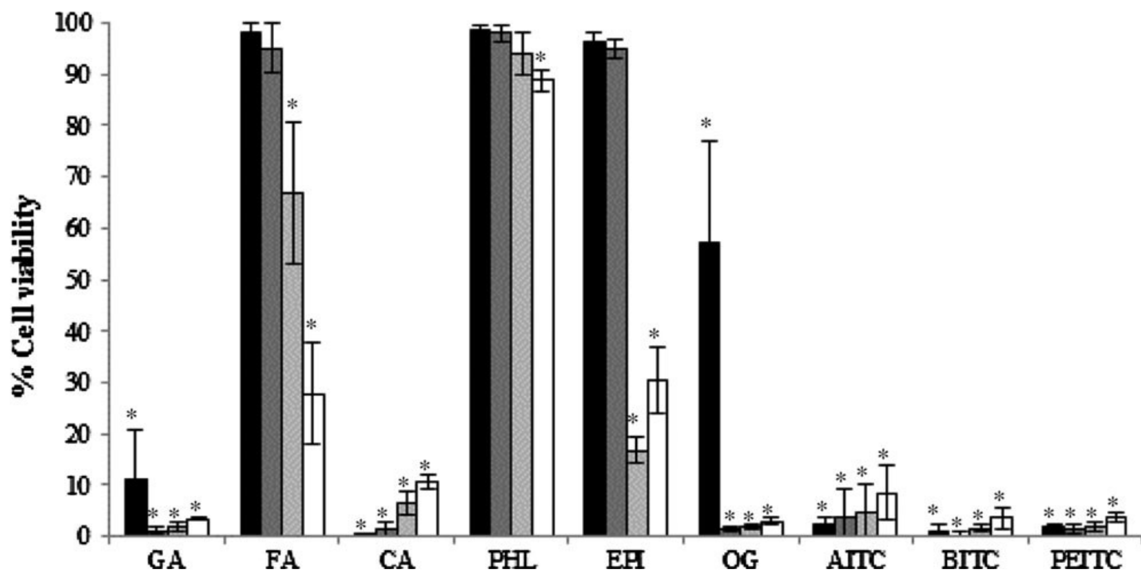


817 Figure 1. Percentage of violacein inhibition (%) by ITCs, AITC (♦), BITC (□) and PEITC
818 (○) at different concentrations (5, 10, 15, 20, 25, 50, 75, 100, 150, 200, 250, 300, 350,
819 500, 750 and 1000 µg ml^{-1}). Mean values ± standard deviation for at least three replicates
820 are illustrated.



831 Figure 2. Percentage of violacein inhibition (%) by phenolic compounds (GA, FA, CA,
832 PHL, EPI and OG) at 1000 µg ml^{-1}. Mean values ± standard deviation for at least three
833 replicates are illustrated. Bars with * are statistically different from the control (($P < 0.05$).

834



835 Figure 3. Percentage of viability (%), obtained with the MTS assay, of the L929 cells
836 exposed to different concentrations (50 (■), 100 (■), 500 (■) and 1000 (□) μg ml^{-1}) of
837 phenolic compounds (GA, FA, CA, PHL, EPI and OG) and ITCs (AITC, BITC, PEITC)
838 for a period of 72 h. Mean values ± standard deviation for at least three replicates are
839 illustrated. Bars with * are statistically different from the control (($P < 0.05$).